# Butyrate-induced apoptosis and cell cycle arrest in bovine kidney epithelial cells: Involvement of caspase and proteasome pathways[1]

## C. J. Li[2] and T. H. Elsasser

Growth Biology Laboratory, Animal and Natural Resources Institute, ARS, USDA, Beltsville, MD 20705

**ABSTRACT:** Beyond their nutritional effect, short-chain fatty acids, especially butyrate, modulate cell differentiation, proliferation, motility, and in particular, they induce cell cycle arrest and apoptosis. A bovine kidney epithelial cell line (Madin-Darby bovine kidney; MDBK) was used to investigate the cell cycle regulatory and apoptotic effects of butyrate. Butyrate not only induced apoptosis but also induced cell cycle arrest at the G1/S boundary and M/G2 in MDBK cells ($P < 0.01$). The cell responses were concentration-dependent ($r^2 = 0.9482$, $P < 0.001$). In examining possible mechanisms for the apoptosis and cell cycle arrest induced by butyrate, the results showed that butyrate treatment activates caspase-3 activities and induces accumulation of acetylated histone. At least two proteins, cdc6 and cdk1, become targeted for destruction on butyrate treatment. These two proteins are downregulated ($P < 0.01$ and $P < 0.05$, respectively) by proteolytic pathways. Moreover, the proteasome inhibitor MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) reverses the cell cycle arrest induced by butyrate, indicating a multiprotein crosstalk wherein the ubiquitination/ proteasome pathway interacted with the caspase-signaling pathway. Because the proteasome inhibitor MG-132 blocked activation of caspase-3, these results functionally locate the proteasome pathway upstream of the caspase pathway. All these results indicate that butyrate functions as both a nutrient and signaling molecule regulating cell growth and proliferation.

Key Words: Apoptosis, Bovine Kidney Epithelial Cell, Butyrate, Caspase-3, Cell Cycle, Proteasome

## Introduction

It is now well established that microbial fermentation in the gastrointestinal tract contributes to the energy balance of all mammalian species (Bergman, 1990; Andoh et al., 2003). Short-chain fatty acids (i.e., acetate, propionate, and butyrate) are formed during microbial fermentation of dietary fiber in the gastrointestinal tract of mammalian species and then are directly absorbed at the site of production. Short-chain fatty acids contribute up to 70% of the energy requirements of ruminants (Bergman, 1990). Although acetate and propionate hold a prominent position in providing energy to ruminant metabolism, butyrate, low in its relative concentrations, seems to be involved in metabolism beyond its role as a nutrient. Roles for butyrate have been established in cell differentiation, proliferation, motility, and, in particular, induction of cell cycle arrest and apoptosis (Scheppach et al., 1995; Gassull and Cabre, 2001; Chen et al., 2003). Apoptosis is a genetically regulated cellular suicide mechanism that plays a crucial role in the development and defense of homeostasis of animals (Johnson, 2002). The mechanism(s) by which butyrate induces cellular differentiation and suppresses growth has not been elucidated. Similarly, the mechanism by which butyrate and other short-chain fatty acids induce the cell cycle regulatory and apoptotic effects and the mechanism by which the decision between cell death and survival is arbitrated are poorly understood. Few definitive studies in cattle, if any, have addressed the capabilities for nutrients to modulate gene expression and proteomic outcomes as a means of arresting metabolic stress. Cell cycle regulatory and apoptotic effects of butyrate and other short-chain fatty acids at the cellular and molecular levels in normal bovine cells have not been studied thus far but would serve as a principle launch point to validate the need for further study of these phenomena in cattle.

## Materials and Methods

### Cell Culture

Madin-Darby bovine kidney epithelial cells (**MDBK**; American Type Culture Collection, Manassas, VA, cata-

log No. CCL-22) were cultured in Eagle's minimal essential medium supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA) in a 25-cm$^2$ flask with medium renewal twice weekly. Cell cultures were maintained in a water-jacked incubator with 5% CO$_2$ at 37°C. Subcultivations were performed when cells attained 80 to 90% confluence, according to the product information supplied by American Type Culture Collection. Cells were used for treatment testing at approximately 50% confluence during the exponential phase of growth.

## Cell Treatments

Sodium butyrate (Calbiochem, San Diego, CA) was prepared as 1 $M$ stock by dissolving it in ultrapure deionized water (tissue culture grade; Advanced Biotechnologies Inc., Columbia, MD). Final concentrations of sodium butyrate treatment ranged from 2.5 to 10 m$M$. Adding up to 10 m$M$ sodium butyrate into cell culture medium did not cause measurable pH change. To determine the extent of proteosome pathway interaction in the effects of butyrate, the proteasome inhibitor MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, Calbiochem) was dissolved in dimethyl sulfoxide and added to the media to a final concentration of 20 μ$M$. Duplicate flasks of cells were used for each of treatment. After 24 h of sodium butyrate, and proteasome inhibitor treatment when indicated, cells were collected by trypsinization. Viability of cells was determined by Trypan Blue stain exclusion (Invitrogen).

## Flow Cytometric Assay of Cells

Flow cytometry analysis is based on measurements of increased DNA content in proliferating cells going through cell cycle phases. Cellular DNA content increases from the original amount of two copies (**2C**) in G1 phase to twice amount of four copies (**4C**) in M/G2 phases, with intermediate DNA content in S-phase cells. To measure the DNA content, cells were stained with a fluorescent dye (propidium iodide; Sigma Chemical Co., St. Louis, MO) that was directly bound to the DNA in the nucleus. Measuring the fluorescent by flow cytometry provided a measure of the amount of dye taken up by the cells and indirectly the amount of DNA content. Cells collected by trypsinization were washed with complete cell culture medium and then with ice-cold PBS (pH 7.4) buffer. Cells were resuspended in PBS buffer and two volumes of ice-cold 100% ethanol were added dropwise into tubes and mixed with cells in suspension by slow vortexing. After ethanol fixation, cells were centrifuged (400 × $g$, 5 min) and washed in PBS buffer once. Cells were resuspended at 10$^6$/mL, and then 50 μg/mL of RNase A (Sigma Chemical Co., St. Louis, MO) was added to each sample and the samples were incubated at 37°C for 30 min. After incubation, 20 μg of propidium iodide were added to each tube for at least 30 min to provide the nuclear signal for fluorescence-activated cell sorting (flow cytometry).

Cell DNA content was analyzed using flow cytometry (FC500, Beckman Coulter, Inc., Palatine, IL), and collected data were analyzed using Cytomics RXP (Beckman Coulter, Inc.). At least 10,000 cells per sample were analyzed.

## Apoptosis Detection

For detection of apoptotic cells by flow cytometry, terminal deoxynucleotidyl transferase nick-end labeling (**TUNEL**) assay was applied according to manufacturer's instruction (apoptosis detection kits, FlowTACS, R & D System, Inc., Minneapolis, MN). During apoptosis, specific Ca-dependent endonucleases cleave genomic DNA and create fragments with double-stranded breaks. Fragmentation of DNA in individual apoptotic cells can be visualized by detection of biotinylated nucleotides incorporated onto the free 3′-hydroxyl residues of these DNA fragments. Terminal deoxynucleotidyl transferase was used to add biotinylated nucleotides to the 3′-ends of the DNA fragments. Cells then were stained with streptavidin-conjugated fluorescein (**FITC**) and analyzed by flow cytometry.

## Preparation of Cell Extracts and Western Blot Analyses

Cells were collected from culture flasks by trypsinization. Cells were then washed first with complete medium with 5% fetal bovine serum and followed by washing with PBS twice. Cells were extracted with 10 volumes of mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL) supplemented with 150 m$M$ NaCl and a protease inhibitor cocktail (protease inhibitor cocktail tablets; Boehringer Mannheim, GmbH, Germany) and then incubated on ice for 10 min before centrifugation (1,500 × $g$, 5 min at 4°C in an Eppendorf [Hamburg, Germany] microfuge) to eliminate the debris. The supernatants were subjected to SDS-PAGE, which was done in 4 to 20% gradient polyacrylamide gel (Invitrogen) under the reducing conditions suggested by Invitrogen. Prestained molecular weight standards were included (SeeBlue-plus2, Invitrogen). Separated proteins were transferred to pure nitrocellulose membrane (Protran, 0.2 μm; Schleicher and Schuell, Dassel, Germany). Western blot analyses were performed using anti-cdc6, anti-acetyl histone3 (**H3**), anti-acetyl-phospho H3 (Cell Signaling Technology, Beverly, MA), anti-caspase 3, and anti-cdk1 (Calbiochem). Secondary antibodies used were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Pierce Biotechnology, Rockford, IL). Membranes were blocked first with 5% of fat-free dry milk in PBS for 1 h and followed by incubation with the antibodies specified (1:2,000 diluted in PBS plus 0.1% Triton 100). Membranes were then washed five times with PBS containing 0.1% Triton 100. Secondary antibodies (1:25,000 diluted with PBS plus 0.1% Triton 100) were added and incubated for 1 h. Membranes were washed

five times with PBS containing 0.1% Triton 100. Immunoblots were exposed to SuperSignal West Pico stable peroxide solution with luminol/enhancer (Pierce Biotechnology) according to the manufacturer's instructions. Western blots were then scanned and analyzed using NIH Image software (National Institutes of Health, Bethesda, MD) to quantify the density of the bands.

Data were statistically analyzed by ANOVA using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA). Data are presented as means ± SEM.

## Results

### Sodium Butyrate Induces Cell Death in Exponentially Growing MDBK Cells

Because butyrate induces different biological effects on different cell types, whether butyrate affects apoptosis and cell proliferation in MDBK cells was investigated. To determine whether sodium butyrate induces cell death and cell cycle arrest, exponentially growing MDBK cells were treated with 2.5 to 10 m$M$ of sodium butyrate for 24 h. All cells, including detached cells, were collected, and aliquots of cells were stained with Trypan blue and counted manually by microscopy. Trypan blue exclusion analysis results indicated that butyrate induces cell death in exponentially growing MDBK cells in a concentration-dependent manner. The effect of butyrate inducing cell death was significant ($P < 0.05$ at 2.5 m$M$; $P < 0.001$ at 5 and 10 m$M$). Flow cytometry analysis by measuring the DNA content of cells confirmed the Trypan blue stain results. By comparing the profiles of normal cells (Figure 1B) and 10 m$M$ butyrate-treated cells (Figure 1C), a pre-G1 population (cells with less than the normal amount of DNA) of approximately 38% of total cells appeared in 10 m$M$ butyrate-treated cells. When detached cells were collected separately and analyzed by flow cytometry, almost 100% of the detached cells belonged to the pre-G1 population, indicating they were nonsurvival cells (Figure 1D). The generation of the pre-G1 population of cells apparently is due to the DNA fragmentation of dead cells induced by butyrate, indicating that apoptosis might occur.

To confirm that cell death was the result of apoptosis, the TUNEL assay was applied. Shown in Figure 2A are the negative control of cells (normal cells without streptavidin-conjugated Fluorescein staining) and the nuclease-generated positive control (normal cells treated with nuclease supplied with the detection kits before terminal deoxynucleotidyl transferase labeling and FITC staining). Approximately 20% of cells were positive for FITC staining in 5 m$M$ butyrate-treated cells and approximately 35% of cells were positive for FITC staining (Figure 2B). The results confirmed that cell death induced by butyrate was indeed due to apoptosis.
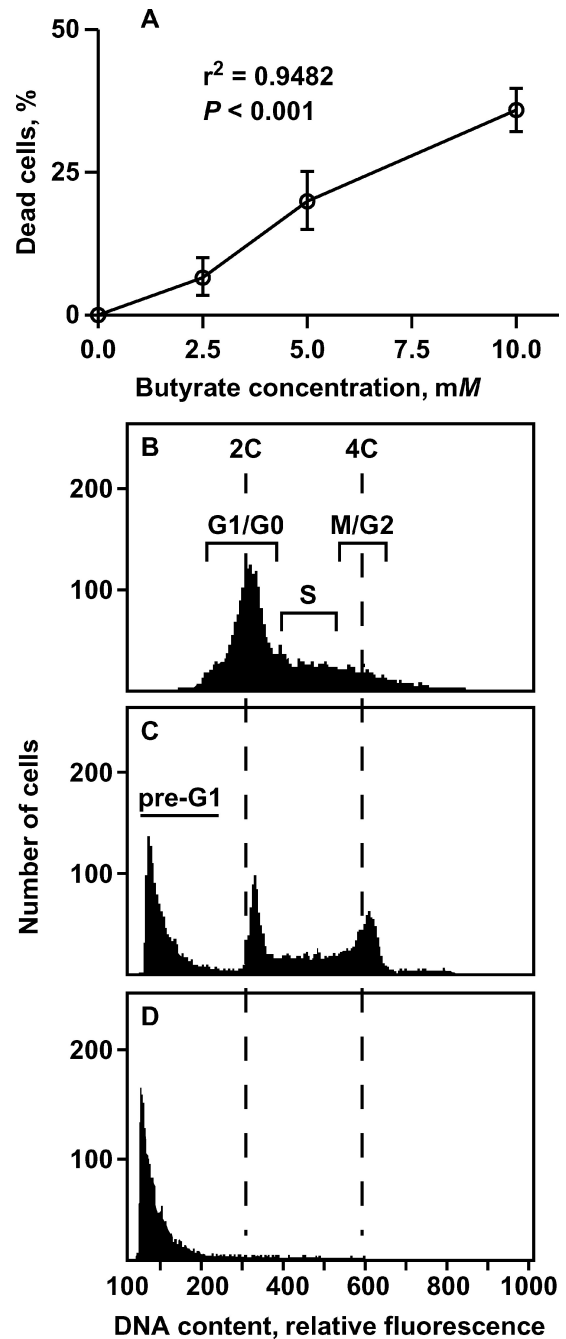


**Figure 1.** Butyrate induces cell death in Madin-Darby bovine kidney (MDBK) epithelial cells. A) Viability of cells determined by trypan blue stain after treatment with different concentrations of butyrate for 24 h (n = 3 per treatment, $P < 0.05$ at 2.5 m$M$, $P < 0.001$ at 5 and 10 m$M$). B) Histogram plot of flow cytometry analysis of MDBK cells sorted by their DNA content. C) Histogram plot of flow cytometry analysis of MDBK cells treated with 10 m$M$ sodium butyrate. D) Detached cells were collected separately and analyzed with flow cytometry. G1/G0 = cells in G1/G0 cell cycle phases; S = cells in S cell cycle phase; M/G2 =cells in M/G2 cell cycle phase; pre-G1 = cells with less than normal amount of DNA content; 2C and 4C = two and four copies of DNA content, respectively.
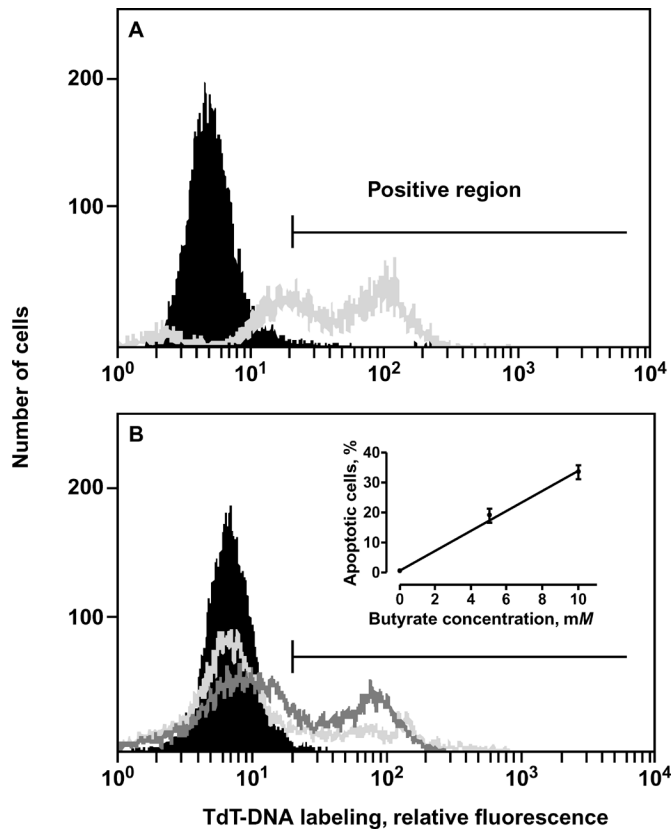
**Figure 2.** Terminal deoxynucleotide transferase nick end labeling (TUNEL) assay confirms apoptosis. Cells were labeled with biotinylated nucleotides to the 3′-ends of the DNA fragments and then were stained with streptavidin-conjugated fluorescein (FITC) and sorted by flow cytometry. A) Overlap of histograms of flow cytometry analysis of normal cell (solid area) without FITC staining (negative control) and nuclease-generated positive control (open area). B) Histograms of 0 m$M$ (solid area). Cells were treated with 5 m$M$ (open area with light-gray line) or 10 m$M$ (open area with dark-gray line) sodium butyrate. Insert shows measurements of TUNEL assay results (n = 3 per treatment; $r^2$ = 0.9572; $P$ < 0.001). TdT = terminal deoxynucleotidyl transferase.

*Proteasome Inhibitor MG-132 Reversed Butyrate-Induced Cell Cycle Arrest and Apoptosis*

To look further into the possibility of cell cycle arrest induced by butyrate, we collected the survival cells (excluding the detached cells population) and analyzed the DNA contents by flow cytometry. As shown in the Figure 3, after butyrate treatment for 24 h, both G1 (cells with 2C DNA contents) and M/G2 populations (cells with 4C DNA contents) of the survival cells were increased and the S-phase cell population (cells falling in between the 2C and 4C DNA contents) was decreased (Figure 3A, B, C). Furthermore, the G1 peak of the butyrate-treated cells displayed a shift in DNA content, indicating an abnormal (aneuploid) DNA content in these cells. Collectively, this suggests that cells were possibly arrested at the G1/S boundary, and DNA replication was blocked at an early stage of S-phase by the butyrate treatment. To determine whether the ubiquitin-proteasome pathway is required for butyrate-induced cell cycle arrest and apoptosis, cells were treated with butyrate and proteasome inhibitor MG-132. After 24 h of treatment, cells were collected and analyzed. When cells were treated with both butyrate and MG-132, cell cycle arrest by butyrate was completely reversed (Figure 3E and F, solid areas).

This experiment was repeated several times and results are consistent. To determine the precise cell cycle proportions, cell cycle analyses of DNA histograms were performed using Cylchred software (CytonetUK, University of Wales College of Medicine, U.K.; Figure 4). The 10 m$M$ butyrate treatment significantly ($P$ < 0.01) increased G1 and M/G2 populations and decreased S-phase cell population. There was no difference between normal cells and cells treated with 10 m$M$ butyrate and 20 µ$M$ proteosome inhibitor MG-132 ($P$ = 0.55).

*Proteasome Inhibitor MG-132 Inhibited the Butyrate-Induced Activation of Caspase 3 and Destruction of cdc6 and cdk1*

The caspase pathway is the major pathway that leads cells into apoptosis (Stegh and Peter, 2001; Gabler et al., 2003). Caspase-3, an executor of cell death, exists as a 34-kDa pro-enzyme protein and becomes activated as a 17- and 12-kDa active caspase when apoptosis is induced. Therefore, activation of caspase-3 is another solid indicator of apoptosis. Using anti-monoclonal antibody, which can detect both pro- and activated caspase-3, Western blot analyses of the cell extracts from the butyrate-treated, butyrate plus MG-132-treated, and normal cells were performed. As shown in Figure 5A and B, 5 and 10 m$M$ butyrate induced activation of caspase-3, whereas MG-132 inhibited the activation of caspase-3. To determine whether butyrate-induced biological effects such as apoptosis and cell cycle arrest alter biochemical properties or relative amounts of proteins required for DNA replication and cell proliferation, and to determine the involvement of the ubiquitin-proteasome pathway in the destruction of cdc6 and cdk1, the levels of cdc6 and cdk1 after butyrate treatment with or without MG-132 treatment were examined. Again, butyrate and trichostatin A treatment induced the destruction of cdc6 and cdk1, whereas the addition of MG-132 to the treatment of butyrate prevented this destruction (Figure 5C, D). In separate experiments, several concentrations of butyrate were used to treat MDBK cells, and the levels of cdc6 and cdk1 were measured. Levels of cdc6 and cdk1 decreased in response to butyrate treatment (cdc6: $r^2$ = 0.9997, $P$ < 0.01; cdc2/csk1: $r^2$ = 0.9971, $P$ < 0.05; Figure 6A, B, C). The loss of cdc6 also paralleled with appearance of faster mobility forms of cdc6, indicating the proteolytic destruction (Figure 6A).
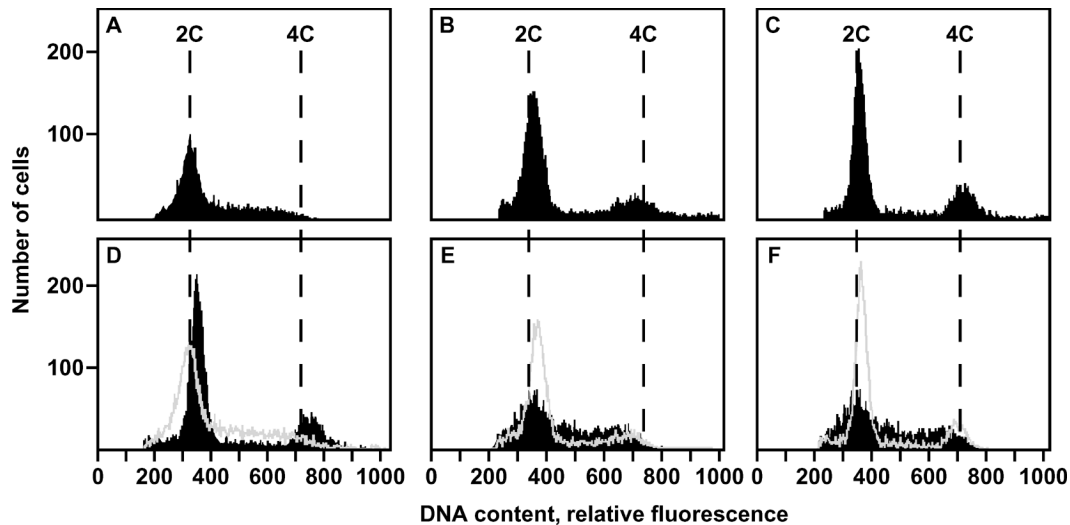
**Figure 3.** Proteasome inhibitor MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) reverses butyrate-induced cell cycle arrest. Survival cells (attached cells) were collected after 24 h of treatment of 5 and 10 m*M* butyrate, cells were fixed, stained with propidium iodide, and analyzed by flow cytometry as described in Materials and Methods. This figure is representative of three independent experiments. A) Normal exponential growing cells without butyrate treatment; B) 5 m*M* butyrate-treated cells; C) 10 m*M* butyrate-treated cells; D) overlapping the histograms of normal cells (open area) and 10 m*M* butyrate-treated cells (solid area) for easy comparison; E) comparison of 5 m*M* butyrate (open area)-treated MDBK cells with 5 m*M* butyrate- and 20 μ*M* proteasome inhibitor MG-132-treated cells (solid area); and F) comparison of 10 m*M* butyrate-treated MDBK cells (open area) with 10 m*M* butyrate- and 20 μ*M* MG-132-treated cells (solid area). 2C and 4C = two and four copies of DNA content, respectively.



**Figure 4.** Cell cycle progressions determined by flow cytometry. Open column = control cells; solid column = 10 m*M* butyrate-treated cells; gray column = 10 m*M* butyrate- and 20 μ*M* proteasome inhibitor MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal)-treated cells (n = 3 per treatment; \*\*\*$P < 0.01$). G1/G0 = cells in G1/G0 cell cycle phases; S = cells in S cell cycle phase; M/G2 = cells in M/G2 cell cycle phases.

## Accumulation of Acetylated H3 and Phosphorylated H3 due to the Butyrate Treatment

It has been known that butyrate functions as a histone deacetylase inhibitor and histone acetylation/deacetylation has been reported to be involved in the regulation of transcription and DNA replication (Johnson, 2002; Chen et al., 2003). To determine whether these biochemical attributes of butyrate were a part of the observed events in MDBK cells, the monoclonal antibodies against the acetyl H3 and acetyl-phospho H3 were used to evaluate the histone deacetylase inhibitory activity of butyrate. As shown in Figure 7, butyrate treatment induced accumulation of acetyl H3 and acetyl and phospho H3. A slower mobility band of histone appeared as the results of the acetylation and phosphorylation (Figure 7A). The results of a Western blot using a monoclonal antibody against the acetyl H3 and an antibody against acetyl-phospho H3 showed that the amount of acetylated H3 increased approximately two- to threefold when cells were treated with 5 and 10 m*M* of butyrate (Figure 7B). When measured by the band density of the Western blot, acetylated and phosphorylated H3 increased three- to fivefold (Figure 7C).

## Discussion

During the last two decades, we have come to realize that lipids function as both energy stores and signaling
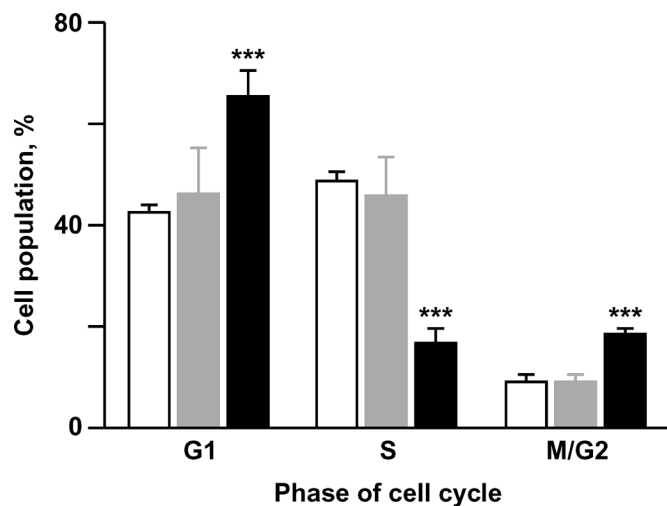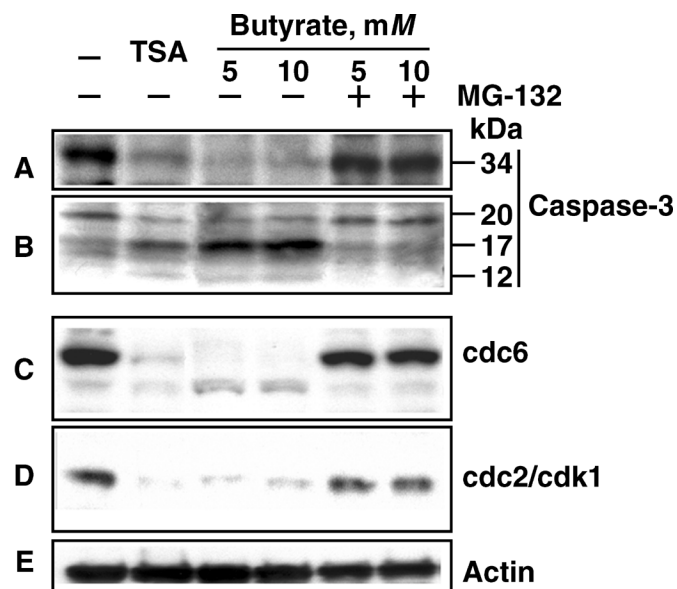
**Figure 5.** Proteasome inhibitor MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) inhibited activation of caspase-3 and destruction of cdc6, cdc2/cdk1. Madin-Darby bovine kidney epithelial cells were extracted after treatments as indicated. Protein samples were subjected to SDS PAGE and Western blotting. This figure is representative of three experiments (n = 3 per treatment). A and B) Pro-caspase-3 (34 kDa) and activated caspase-3 (20-, 17-, 12-kDa bands). C and D) The same blot membrane was stripped and reblotted with anti cdc6 and cdc2/cdk1 antibodies. E) Loading control. MG-132 = proteasome inhibitor, 20 $\mu M$. TSA = 100 n$M$ trichostatin A.



**Figure 6.** Butyrate treatment induced the destruction of cell cycle regulatory proteins cdc6 and cdc2/cdk1. Butyrate-treated cells were extracted and proteins were separated on SDS PAGE. Western blot analyses were performed with antibody against cdc6 and cdc2/cdk1. A) Western blotting of cdc6 and cdc2/cdk1. B and C) Western blots from three experiments were quantified with NIH Image software (National Institutes of Health, Bethesda, MD). The relative densities were measured and corrected with the protein density. Density of control band is presented as 100% (n = 3 per treatment). TSA = trichostatin A.

molecules. They play pivotal roles in regulating a wide variety of cellular processes. As a particular group of lipids, short-chain fatty acids have been studied intensively (Hague et al., 1996; Andoh et al., 2003). Short-chain fatty acids are a major energy source in ruminants and contribute up to 70% of their energy requirements (Sharp et al., 1982; Siciliano-Jones and Murphy, 1989; Bergman, 1990). Of these short-chain fatty acids, butyrate is the major source of energy for colonocytes (Roediger, 1980; Bugaut, 1987). Short-chain fatty acids are organic acids produced by anaerobic fermentation of dietary fiber in the gastrointestinal tract of mammalian species and then directly absorbed at the site of production. Short-chain fatty acids, especially butyrate, are important nutrients and function as signaling molecules. The molecular mechanisms involved in biochemical and molecular actions of these nutrients remain largely unknown. Few definitive studies in cattle, if any, have addressed the capabilities of short-chain fatty acids as signaling molecules to modulate gene expression and the proteometric outcomes at cellular and molecular levels.

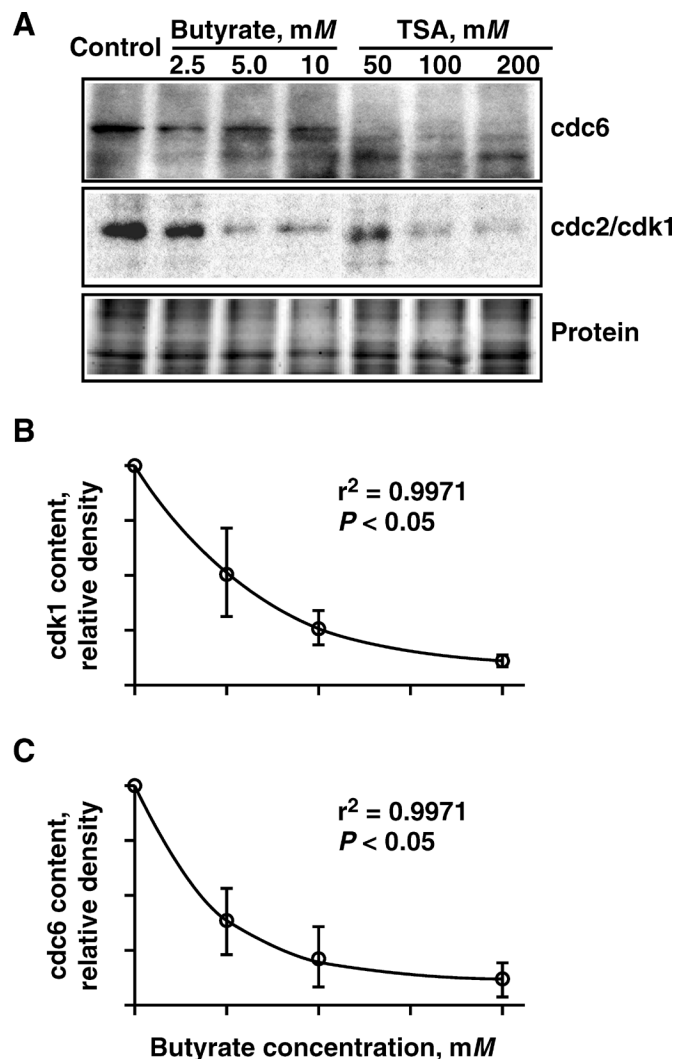All three major components of short-chain fatty acids (acetate, propionate, and butyrate) induce apoptosis and inhibit cell proliferation. However, butyrate was the most potent in inducing apoptosis and inhibition of cell proliferation (Hague and Paraskeva, 1995; Emenaker et al., 2001). Therefore, butyrate was investigated for its biological effects in an established cattle cell line MDBK in this study. A concentration range of 2.5 to 10 m$M$ of butyrate was selected in all experiments for two major reasons. First, historically, most of the experiments reported on cancer or other cells lines used similar concentration range. Second, in ruminants such as sheep and cattle, short-chain fatty acids contribute up to 70% of their caloric requirements. Rates of short-
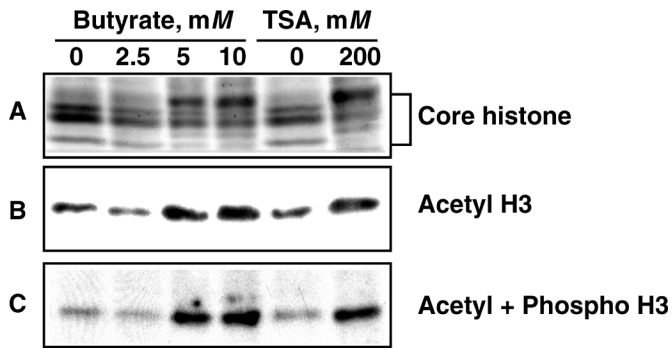
**Figure 7.** Butyrate-induced hyperacetylation of histone3 (H3). This figure is representative of three experiments (n = 3 per treatment). Equal amounts of protein from different samples were separated by SDS PAGE on two identical 4 to 20% polyacrylamide gradient gels. One gel was stained with SimpleBlue (Invitrogen, Carlsbad, CA), and one was transferred to a membrane for Western blotting. A) SimpleBlue-stained gel. B) The result of Western blotting with monoclonal anti- acetyl H3 antibody. C) The result of Western blotting with monoclonal anti-acetyl-phospho H3 antibody. TSA = trichostatin A.

chain fatty acid production and absorption in ruminants are much higher than in other animals and human, which has been calculated to be approximately 5 mol/kg DMI (Bergman, 1990). The principal short-chain fatty acids in either the rumen or large intestine are acetate, propionate, and butyrate, and they are produced in a ratio varying from approximately 75:15:10 to 40:40:20 (Bergman, 1990). The concentrations of short-chain fatty acids in the rumen are highly variable, and the total amount present usually fluctuates between 60 to 150 m$M$. Butyrate concentrations in the digestive tract and blood of sheep are established between 0.5 to 13 m$M$ (Bergman, 1990). Therefore, concentrations of 2.5 to 10 m$M$ of butyrate used in the present study are in a reasonable range to test its biological effects. It is also very unlikely that butyrate causes cell death due to its acidity. Butyrate is a weak acid with a p$K$ of 4.82. A 1 $M$ solution of sodium butyrate has a pH of approximately 6.5 to 7.0. Adding 10 m$M$ sodium butyrate to culture medium does not cause measurable pH change. Results of TUNEL assay and activation of caspase-3 upon butyrate treatment also strongly argue that cell death is due to apoptosis.

An important question asked in this study is whether normal bovine cells in a standard cell culture condition are sensitive to the growth inhibitory effects of butyrate. The presented data showed that sodium butyrate could induce apoptosis and cell cycle arrest in MDBK cells. Up to approximately 38% of cells become apoptotic after 24 h of treatment with 10 m$M$ of butyrate. In addition, the data showing the accumulation of cells with both 2C and 4C DNA contents in survival cells suggests that butyrate blocks the cell cycle at two distinct stages, G1 and M/G2. These findings are consistent with the data reported in the studies on most cancer cell lines (Coradini et al., 2000; Qiu et al., 2000). Because previous studies documented that butyrate induces apoptotic cell death by a process that is dependent on the inhibition of histone deacetylase and new protein synthesis (Medina et al., 1997; Chen et al., 2003), the correlation between apoptosis/cell cycle arrest and histone hyperacetylation was also examined. The results clearly linked the accumulation of hyperacetylated histone-3 to the treatment of butyrate; however, more studies are needed for better understanding of the relation between hyperacetylation and butyrate.

During the process of apoptosis, certain proteins become the targets of destruction. To analyze cell cycle control in bovine cells using butyrate, its molecular target must be determined. Accumulation of cells with 2C and 4C DNA contents suggests inhibition by butyrate of cell cycle at G1 and M/G2 phases and suggests that a common responding element in genes responsive to the treatment of butyrate is required for progression of both phases G1 and M/G2. This observation is also consistent with the previous report that the inhibition of G1 progression by butyrate is not restricted to a specific mitogenic signaling pathway (Charollais et al., 1990), but may also include the inhibitory effect on initiation of DNA replication. To determine the epigenetic effects of physiologic concentrations of butyrate on normal bovine cells, the cellular level of proteins, which are the putative targets of butyrate-induced cell cycle arrest and apoptosis, was examined. The cdc6, a protein that has been shown to be rate limiting for initiation of DNA replication in a number of eukaryotic cell lines, is essential for the assembly of the prereplication complex (Sun et al., 2002). Prereplication complexes are assembled in early G1 (Li et al., 2000; Li and DePamphilis, 2002) and are maintained by cdc6 and other prereplication complex proteins until initiation of DNA replication occurs. At least four roles have now been recognized for cyclin A-activated Cdk1 protein kinases in regulating cell cycle events. First, Cdk2/cyclin A is responsible for activating prereplication complexes at the beginning of the S-phase to begin DNA synthesis (Coverley et al., 2002). Second, Cdk2/cyclin A inhibits assembly of new prereplication complexes during the S-phase (Coverley et al., 2002) by inactivating cdc6 (Jiang et al., 1999). Third, Cdk2/cyclin A is required for the G2 to M-phase transition (Furuno et al., 1999). Finally, Li et al. (2004) revealed recently that Cdk1/cyclin A is required for preventing Orc1 in mammals or ORC in *Xenopus* from binding to chromatin during mitosis. Here, the results show that targeted destruction of cdc6 and cdc2/cdk1 may be also involved in the apoptosis and cell cycle arrest induced by butyrate. It is also very likely that the proteolytic destruction of these two proteins is part of DNA damage checkpoint responses that inhibit initiation of DNA replication (Blanchard et al., 2002; Castedo et al., 2002). These results suggest that activation of caspase-3 directly correlates with the destruction of cdc6 and cdc2/cdk1, and

therefore strongly indicating that these two proteins may be the casualties of apoptosis progression. Moreover, these results are consistent with the growing body of evidence suggesting that disruption of the coordinate between regulation of DNA synthesis and cyclin-dependent kinase activity is an important feature of apoptosis. This disruption causes the unscheduled activation of cyclin-dependent kinases in cells with unreplicated or partially replicated chromosomes (Guo and Hay, 1999). This may well explain the shift of G1 peak with abnormal (aneuploid) DNA content of MDBK cells after treatment with butyrate. The other finding in this report is that proteasome inhibitor MG-132 can reverse butyrate-induced cell cycle arrest and apoptosis. This suggests that the ubiquitin-proteasome pathway is also involved in the butyrate induced cellular processes. In recent years, much attention has been given to mechanisms that regulate protein degradation by the ubiquitin-proteasome. Since the discovery of the ubiquitin-proteasome pathway, it has become clear that proteolysis of cellular proteins is a highly complex, temporally controlled and tightly regulated process. It is carried out by a complex cascade of enzymes and displays a high degree of specificity (Ciechanover, 1998; Glickman and Ciechanover, 2002), which plays major roles in a variety of basic pathways during cell life and death as well as in health and disease. Even though the involvement of the proteasome in apoptosis and cell cycle regulation has been established, the exact function and mechanisms of its action remains to be elucidated. Although many studies used proteasome inhibitors to demonstrate the induction of apoptosis, others found that those agents inhibited programmed cell death (Orlowski, 1999; Wojcik, 2002). The results reported in this study showed that proteasome inhibitor MG-132 inhibits the activation of caspase-3, suggesting the possibility that proteasomes act upstream of the caspase cascade in the apoptotic process (Orlowski, 1999), whether that activity results in the promotion or inhibition of apoptosis and cell cycle arrest. The proteasome pathway is also strongly implicated in the cell cycle regulation (King et al., 1996; Hershko, 1997). The results in this report show that inhibition of proteasome activity results in reversal of cell cycle arrest and is involved in the targeted destruction of two cell cycle regulators (cdc6 and cdk1). It is reasonable to conclude that the proteasome pathway regulates the cell cycle directly through the specific and programmed degradation of cell cycle regulators or indirectly through interaction with caspase pathway. In conclusion, all of these results suggest that butyrate functions as both a nutrient and signal molecule regulating the cell growth and proliferation. In addition, butyrate signals involve multiple genes, multiple protein interactions, and multiple signal transduction pathways cross talking.

Because short-chain fatty acids are common nutrients, understanding their important biological functions will certainly help us to understand critical control points in cell life cycle functions that could lead to im-

provements in the efficient production of food animals. Short-chain fatty acid-induced apoptosis and cell cycle arrest may affect growth slumps of young cattle during periods of naturally encountered stresses, such as the weaning period, when production and uptake of short-chain fatty acids are significantly increased. Minimizing the duration of growth slumps has the potential to affect the industry significantly. In addition, MDBK as an established bovine cell line with inducible apoptosis and cell cycle regulatory events certainly is an ideal and invaluable tool for functional genomic studies on homeostasis in animals.

## Literature Cited

Andoh, A., T. Tsujikawa, and Y. Fujiyama. 2003. Role of dietary fiber and short-chain fatty acids in the colon. Curr. Pharm. Des. 9:347–358.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70:567–590.

Blanchard, F., M. E. Rusiniak, K. Sharma, X. Sun, I. Todorov, M. M. Castellano, C. Gutierrez, H. Baumann, and W. C. Burhans. 2002. Targeted destruction of DNA replication protein cdc6 by cell death pathways in mammals and yeast. Mol. Biol. Cell. 13:1536–1549.

Bugaut, M. 1987. Occurrence, absorption and metabolism of short chain fatty acids in the digestive tract of mammals. Comp. Biochem. Physiol. B 86: 439–472.

Castedo, M., J. L. Perfettini, T. Roumier, and G. Kroemer. 2002. Cyclin-dependent kinase-1: Linking apoptosis to cell cycle and mitotic catastrophe. Cell Death Differ. 9:1287–1293.

Charollais, R. H., C. Buquet, and J. Mester. 1990. Butyrate blocks the accumulation of cdc2 mrna in late g1 phase but inhibits both the early and late g1 progression in chemically transformed mouse fibroblasts bp-a31. J. Cell Physiol. 145:46–52.

Chen, J. S., D. V. Faller, and R. A. Spanjaard. 2003. Short-chain fatty acid inhibitors of histone deacetylases: Promising anticancer therapeutics? Curr. Cancer Drug Targets 3:219–236.

Ciechanover, A. 1998. The ubiquitin-proteasome pathway: On protein death and cell life. EMBO J. 17:7151–7160.

Coradini, D., C. Pellizzaro, D. Marimpietri, G. Abolafio, and M. G. Daidone. 2000. Sodium butyrate modulates cell cycle-related proteins in ht29 human colonic adenocarcinoma cells. Cell Prolif. 33:139–146.

Coverley, D., H. Laman, and R. A. Laskey. 2002. Distinct roles for cyclins e and a during DNA replication complex assembly and activation. Nat. Cell Biol. 4:523–528.

Emenaker, N. J., G. M. Calaf, D. Cox, M. D. Basson, and N. Qureshi. 2001. Short-chain fatty acids inhibit invasive human colon cancer by modulating upa, timp-1, timp-2, mutant p53, bcl-2, bax, p21 and pcna protein expression in an in vitro cell culture model. J. Nutr. 131:3041S–3046S.

Furuno, N., N. den Elzen, and J. Pines. 1999. Human cyclin a is required for mitosis until mid prophase. J. Cell Biol. 147:295–306.

Gabler, C., J. R. Kalden, and H. M. Lorenz. 2003. The putative role of apoptosis-modified histones for the induction of autoimmunity in systemic lupus erythematosus. Biochem. Pharmacol. 66:1441–1446.

Gassull, M. A., and E. Cabre. 2001. Nutrition in inflammatory bowel disease. Curr Opin Clin Nutr. Metab. Care 4:561–569.

Glickman, M. H., and A. Ciechanover. 2002. The ubiquitin-proteasome proteolytic pathway: Destruction for the sake of construction. Physiol. Rev. 82:373–428.

Guo, M., and B. A. Hay. 1999. Cell proliferation and apoptosis. Curr. Opin. Cell Biol. 11: 745–752.

Hague, A., A. J. Butt, and C. Paraskeva. 1996. The role of butyrate in human colonic epithelial cells: An energy source or inducer of differentiation and apoptosis? Proc. Nutr. Soc. 55:937–943.

Hague, A., and C. Paraskeva. 1995. The short-chain fatty acid butyrate induces apoptosis in colorectal tumour cell lines. Eu.r J. Cancer Prev. 4:359–364.

Hershko, A. 1997. Roles of ubiquitin-mediated proteolysis in cell cycle control. Curr. Opin. Cell Biol. 9:788–799.

Jiang, W., N. J. Wells, and T. Hunter. 1999. Multistep regulation of DNA replication by cdk phosphorylation of hscdc6. Proc. Natl. Acad. Sci. USA 96:6193–6198.

Johnson, I. T. 2002. Anticarcinogenic effects of diet-related apoptosis in the colorectal mucosa. Food Chem. Toxicol. 40:1171–1178.

King, R. W., R. J. Deshaies, J. M. Peters, and M. W. Kirschner. 1996. How proteolysis drives the cell cycle. Science 274:1652–1659.

Li, C. J., J. A. Bogan, D. A. Natale, and M. L. DePamphilis. 2000. Selective activation of pre-replication complexes in vitro at specific sites in mammalian nuclei. J. Cell Sci. 113 (Part 5): 887–898.

Li, C. J., and M. L. DePamphilis. 2002. Mammalian orc1 protein is selectively released from chromatin and ubiquitinated during the s-to-m transition in the cell division cycle. Mol. Cell Biol. 22:105–116.

Li, C. J., A. Vassilev, and M. L. DePamphilis. 2004. Role for cdk1 (cdc2)/cyclin a in preventing the mammalian origin recognition complex's largest subunit (orc1) from binding to chromatin during mitosis. Mol. Cell Biol. 24:5875–5886.

Medina, V., B. Edmonds, G. P. Young, R. James, S. Appleton, and P. D. Zalewski 1997. Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin a (inhibitors of histone deacetylase): Dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. Cancer Res. 57:3697–3707.

Orlowski, R. Z. 1999. The role of the ubiquitin-proteasome pathway in apoptosis. Cell Death Differ. 6: 303–313.

Qiu, L. A. Burgess, D. P. Fairlie, H. Leonard, P. G. Parsons, and B. G. Gabrielli. 2000. Histone deacetylase inhibitors trigger a g2 checkpoint in normal cells that is defective in tumor cells. Mol. Biol. Cell 11:2069–2083.

Roediger, W. E. 1980. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. Gut 21:793–798.

Scheppach, W., H. P. Bartram, and F. Richter. 1995. Role of short-chain fatty acids in the prevention of colorectal cancer. Eur. J. Cancer 31A:1077–1080.

Sharp, W. M., R. R. Johnson, and F. N. Owens. 1982. Ruminal VFA production with steers fed whole or ground corn grain. J Anim. Sci. 55:1505–1514.

Siciliano-Jones, J., and M. R. Murphy. 1989. Production of volatile fatty acids in the rumen and cecum-colon of steers as affected by forage: Concentrate and forage physical form. J. Dairy Sci. 72:485–492.

Stegh, A. H., and M. E. Peter. 2001. Apoptosis and caspases. Cardiol. Clin. 19:13–29.

Sun, W. H., T. R. Coleman, and M. L. DePamphilis. 2002. Cell cycle-dependent regulation of the association between origin recognition proteins and somatic cell chromatin. EMBO J. 21:1437–1446.

Wojcik, C. 2002. Regulation of apoptosis by the ubiquitin and proteasome pathway. J. Cell Mol. Med. 6:25–48.